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Differential Effects of a Topoisomerase I Inhibitor on Dioxin Inducibility and High-Level Expression of the Cytochrome P450IA1 Gene

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SUMMARY

The basic helix-loop-helix containing dioxin receptor mediates dioxin signal transduction. The ligand-activated receptor complex binds to specific sequences termed xenobiotic response elements and regulates transcription of target genes such as the gene for cytochrome P450IA1. This study demonstrates that induction of cytochrome P450IA1 and P450IB1 gene expression by a dioxin receptor ligand is repressed by camptothecin, an inhibitor of the topoisomerase I enzyme. However, a transiently transfected reporter construct under control of an xenobiotic response element-containing promoter was not affected by the topoisomerase inhibitor. In agreement with this observation, ligand-dependent activation of the dioxin receptor to its DNA-binding form is not altered by camptothecin as analyzed by electrophoretic mobility shift assay. Moreover, the inhibitory effect of camptothecin cannot be exerted once the P450IA1 gene has been activated. These results imply that topoisomerase I activity is necessary for the primary P450IA1 induction response, possibly involving dioxin-dependent alterations in chromatin structure of the P450IA1 promoter.

The *CYPIA1* gene belongs to a subfamily of cytochrome P450s that are induced in response to environmental pollutants, such as halogenated aromatic hydrocarbons, including dioxin. This gene encodes a protein that has important roles in the metabolic activation of polycyclic aromatic hydrocarbons and heterocyclic amines, respectively, to their ultimate carcinogenic metabolites (reviewed in Ref. 1). The mechanism by which dioxin transcriptionally activates the *CYPIA1* gene involves binding of this ligand to the dioxin receptor (also called the Ah receptor). The dioxin receptor belongs to the basic HLH family of transcription factors (2, 3) and shows homologies to a protein termed Arnt (4) as well as to the mouse hypoxia-induced factor (5) and the *Drosophila* proteins Per and Sim (4). The region of similarity to these proteins contains two direct repeats of ~50 amino acids and is called the PAS (Per-Arnt-Sim) domain.

The nonactivated dioxin receptor is complexed with hsp90 (reviewed in Ref. 6). hsp90 appears to maintain the ligand binding conformation of the dioxin receptor because the hsp90-free form of the receptor shows reduced affinity for dioxin (7). On ligand binding, the receptor is activated to its DNA-binding form by release from hsp90 and heteromerization with Arnt. This activated receptor complex specifically binds XREs upstream of the *CYPIA1* gene (8). The HLH and the PAS domains mediate heteromerization between the dioxin receptor and Arnt (9, 10), and the basic domain shows sequence-specific DNA binding properties (9, 11).

It is likely that transcription requires topological alterations of DNA structure because the packing of DNA into chromatin confers general repression on the expression of genes (12). The major DNA-relaxing enzyme in the nucleus is topoisomerase I, and immunoreactivity specific for this protein localizes to transcriptionally active genes (13). The reaction mechanism by which topoisomerase I relaxes negatively supercoiled DNA involves making a transient break in one strand of the DNA, pulling the other strand through the gap, and then sealing the gap. When topoisomerase I binds to DNA, it forms a stable complex in which the 3′-phosphate end is covalently linked to a tyrosine residue in the enzyme (14). Camptothecin is a cytotoxic alkaloid with antineoplastic action that targets topoisomerase I with high specificity *in vivo* (15). In yeast cells, the toxic effect of the drug is completely dependent on the presence of the enzyme (16). Moreover, mammalian cells can show resistance to the drug that

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ABBREVIATIONS: P450IA1, cytochrome P450IA1; *CYPIA1*, cytochrome P450IA1 gene; P450IB1, cytochrome P450IB1; HLH, helix-loop-helix; dioxin, 2,3,7,8-tetrachlorodibenzo-*p*-dioxin; XRE, xenobiotic response element; hsp90, heat-shock protein, 90 kDa; TCDF, 2,3,7,8-tetrachlorodibenzofuran; DMSO, dimethylsulfoxide; ANF, α-naphthoflavone; CAT, chloramphenicol acetyltransferase; EMSA, electrophoretic mobility shift assay; kb, kilobase(s); GAPDH, glyceraldehyde-3-phosphate dehydrogenase.

often correlates to the presence of a mutation in the topoisomerase I gene (17).

The effect of basic HLH factors on chromatin structure and/or dependence on chromatin conformation is not known in any greater detail, but c-*myc* and *max* have been shown to bend DNA as well as having differential binding to nucleosomal DNA *in vitro* (18). In a similar manner, the activated dioxin receptor complex can induce bending of DNA *in vitro* (19). Furthermore, activation of transcription by dioxin is accompanied by a change in chromatin structure as assessed by DNase I hypersensitivity analysis of the *CYPIA1* gene (20, 21). Not only does dioxin treatment of target cells lead to increased nuclease sensitivity at the receptor binding XRE sequences, but also the region proximal to the transcriptional start site becomes more accessible to DNase I and restriction enzymes (20–22). Moreover, polymerase chain reaction-mediated *in vivo* footprinting studies indicate that the dioxin-induced chromatin changes are accompanied by occupation of XRE elements (23, 24). In the present study, we investigated the role of topoisomerase I activity with regard to *CYPIA1* induction and expression by using the specific inhibitor camptothecin.

Materials and Methods

Cell culture and treatments. Human keratinocytes were isolated from adult donors and cultured as described previously (25) in MCDB 153 medium at a final concentration of 70 μM Ca^{2+} supplemented with 0.1 mM ethanolamine, 0.1 mM phosphoethanolamine, 0.4 μg/ml hydrocortisone, 10 ng/ml epidermal growth factor, 5 μg/ml insulin, 100 μg/ml bovine pituitary extract (Pelfreeze), 100 μg/ml streptomycin, 100 IU/ml penicillin (GIBCO), and 0.25 μg/ml fungizone (GIBCO). Cells at the third to fifth passages were routinely used for experiments. To maximize the cytochrome P450IA1 induction response in keratinocytes, the medium was switched to contain 2 mM Ca^{2+}, and 5% (v/v) fetal bovine serum (Hyclone) was substituted for bovine pituitary extract 48 hr before treatment (25). The human hepatoma cell line HepG2 was grown in Dulbecco's modified Eagle's medium (GIBCO) supplemented with 10% fetal calf serum (GIBCO), 100 μg/ml streptomycin, 100 IU/ml penicillin, and 0.25 μg/ml fungizone. Cultures were treated with TCDF (Cambridge Isotope Laboratories), camptothecin, or α-naphthoflavone (Serva) dissolved in DMSO, whereas control cultures received solvent only, not exceeding a final concentration of 0.2% (v/v). All chemicals, media, and growth factors were purchased from Sigma Chemical Co. unless stated otherwise.

RNA isolation and Northern blot analysis. Total RNA was isolated using acid-phenol extraction as described, and cytochrome P450IA1 mRNA levels were analyzed by Northern blot analysis using standard procedures (26). ^{32}P-labeled probes for cytochromes P450IA1 (27) and P450IB1 (28) and GAPDH mRNA (29) were generated by a random priming procedure. RNA was fractionated through formaldehyde-agarose gels, blotted onto nylon membranes, and UV cross-linked. The filters were subsequently prehybridized, hybridized, and washed according to standard procedures (26) before autoradiography.

Transfection and transient expression assays. Keratinocytes were incubated overnight in MCDB 153 medium containing 70 μM Ca^{2+}, 1.2 μg of cationic liposomes/cm^2 (Lipofectin, Bethesda Research Laboratories), and the indicated amount of plasmid DNA for 16–20 hr. In transient transfection experiments, reporter gene constructs were used that contained a minimal XRE-driven promoter (pTX.DIR; 30) or the phMC7.6 construct, which contains a DNA fragment carrying a part of the first intron, the first exon, and 5′ flanking DNA sequence of ~7.6 kb of the human cytochrome P450IA1 gene fused to the CAT reporter gene (31). After incubation with DNA, the medium was changed to contain 5% serum and 2.0 mM Ca^{2+} for 24 hr, and the cells were subsequently treated with 50 nM TCDF in the presence or absence of 1 μM camptothecin or DMSO for an additional 24–48 hr before harvest. The luciferase and CAT assays were performed as described previously (26) with the modification that the cells were lysed on ice by a brief sonication. Luciferin was purchased from BioThema, and [^{14}C]chloramphenicol was purchased from Amersham.

Cell extract preparation and EMSA. Cells were treated with 50 nM TCDF in the absence or presence of 1 μM camptothecin for the indicated period of time. Control cultures received DMSO. Nuclei were prepared and protein was extracted as described previously (32). DNA binding reactions were assembled in a total volume of 20 μl with 10 μg of nuclear protein at a final concentration of 25 mM 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid, pH 7.9, 0.2 mM EDTA, 75 mM KCl, 2 mM $MgCl_2$, 1 mM dithiothreitol, 0.1 mM phenylmethylsulfonylfluoride, 100 kIU/ml aprotinin (Bayer), 10 μg/ml leupeptin, 5 μM pepstatin (Boehringer), 5% glycerol, 4% Ficoll, 100 μg/ml poly(dI-dC) (Pharmacia), and 12.5 μg/ml poly(dA-dT) (Pharmacia) using a double-stranded, ^{32}P-labeled oligonucleotide as a probe, the sequence of which corresponded to −968 to −997 of the human *CYPIA1* gene (5′-CTCCGGTCCTTCTCACGCAACGCCTGGGCA-3′, sense orientation). In competition experiments, a 100-fold molar excess of unlabeled oligonucleotide was used. Unspecific competitor DNA is described elsewhere (25). When dioxin receptor or Arnt antisera was used (9), 10 μg of nuclear protein was preincubated with the diluted antiserum (1:10) for 20 min at room temperature before the DNA-binding reaction. The antisera were raised against the amino-terminal part of the mouse dioxin receptor (33) and the human Arnt protein (4), respectively. DNA/protein complexes were separated under nondenaturing conditions on a 4% polyacrylamide gel (29:1) run in 1× TGE (50 mM Tris, 2.7 mM EDTA, and 380 mM glycine) at 4°.

Results

Northern blot analyses of human keratinocytes showed that the topoisomerase I inhibitor camptothecin potently suppressed P450IA1 mRNA steady state-level induction by the dioxin receptor ligand TCDF. Inhibition of P450IA1 mRNA expression was dose dependent, and maximal inhibition was achieved at a concentration of 2.5 μM (Fig. 1A). Fig. 1B shows that this effect was not specific to keratinocytes because camptothecin also inhibited the P450IA1 induction response in the human hepatoma cell line HepG2. In these cells, maximal inhibition was obtained at 20 μM. The treatments did not influence expression of GAPDH mRNA, which was used as a control. These concentrations of camptothecin have been shown to be within the observed range for inhibition of expression of genes such as c-*fos* and tyrosine aminotransferase (34, 35). Analysis of the time course of P450IA1 repression by camptothecin revealed that the TCDF response was reduced already 4hr after 4-hr cotreatment with camptothecin and that this inhibition remained throughout the 24-hr experiment (Fig. 1C).

To determine whether topoisomerase I activity was necessary for dioxin induction of other response genes, we analyzed the effect of camptothecin on P450IB1 induction. This recently described cytochrome P450 is induced by dioxin in several cell types, including keratinocytes (36, 28). We have used this gene to investigate whether the inhibitory effect of camptothecin was specific to the P450IA1 gene. As can be seen in Fig. 2, camptothecin treatment potently inhibited TCDF induction of P450IB1.

EMSA was performed to examine whether camptothecin

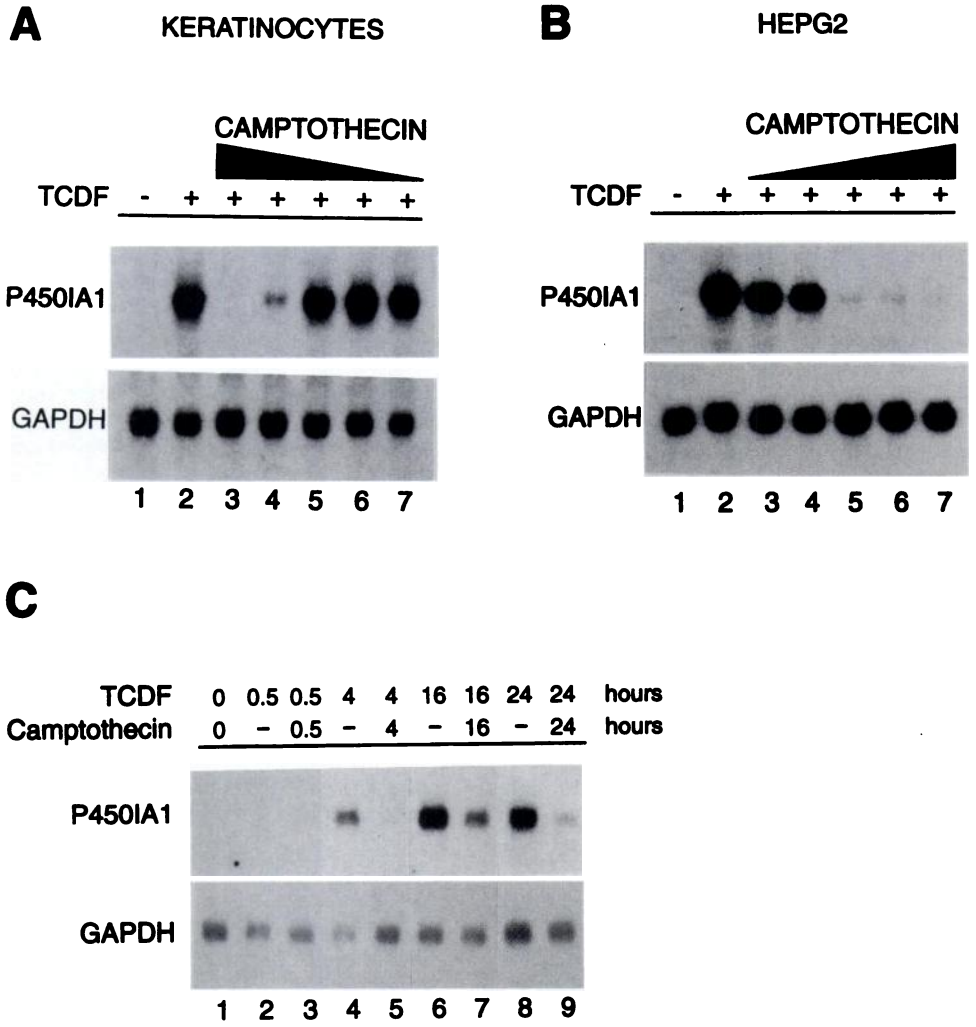

Fig. 1. Inhibition of P450IA1 mRNA induction response by a topoisomerase I inhibitor. Representative Northern blot analyses of P450IA1 and GAPDH dehydrogenase mRNA steady state levels are shown. A, Human keratinocytes were simultaneously treated with solvent alone (DMSO) or 50 nM TCDF in the presence or absence of camptothecin (2.5, 0.63, 0.16, 0.04, and 0.01 μM, respectively) for 4 hr before harvest. B, Total RNA from the human hepatoma cell line HepG2 (*HEPG2*) treated with 50 nM TCDF and increasing concentrations of camptothecin (1, 5, 20, 40, and 80 μM, respectively) for 24 hr was analyzed by Northern blot analysis. C, Time course of repression of P450IA1 by camptothecin. Total RNA from human keratinocytes that had been treated with 50 nM TCDF in the absence or presence of 1 μM camptothecin for 0.5, 4, 16, and 24 hr, respectively, was analyzed. Cytochrome P450IA1 and control mRNA levels were assayed by RNA blot analysis of 8 μg of total cellular RNA/lane.

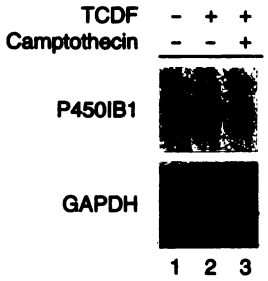

Fig. 2. Camptothecin inhibition of TCDF-induced cytochrome P450IB1 in human keratinocytes. Northern blot analysis showing P450IB1 and GAPDH mRNA levels after treatment with solvent alone or with 50 nM TCDF in the absence or presence of 1 μM camptothecin for 16 hr. Eight micrograms of total RNA were loaded in each lane.

treatment *in vivo* could affect the levels of DNA-binding activity of the dioxin receptor as assayed *in vitro*. Nuclear extracts were prepared from keratinocytes treated with vehicle alone or camptothecin for 1 hr before the addition of TCDF for an additional hour. TCDF-treated cells showed an induced DNA/protein complex that harbored both the dioxin receptor and Arnt, which were identified by antibodies directed against the proteins. Camptothecin treatment did not affect the formation of this receptor complex with the ^{32}P-labeled XRE probe (Fig. 3). Taken together, these results imply that topoisomerase activity was necessary for TCDF-induced P450IA1 expression but did not interfere with activation of the dioxin receptor to its DNA-binding form.

To investigate further the role of topoisomerase I, we transiently transfected a reporter gene construct driven by the P450IA1 promoter region up to ~−7.6 kb (31). This plasmid construct showed 3-fold induction of CAT activity by TCDF. Camptothecin treatment alone did not stimulate reporter gene activity, whereas an 18-fold induction was obtained when cells were treated with both TCDF and camptothecin (Fig. 4A). This indicates that topoisomerase I activity is not necessary for the dioxin receptor to exert its transactivating function; rather, the topoisomerase I inhibitor appeared to potentiate this response in transient transfection assays. In agreement with these results, dioxin induction of a minimal

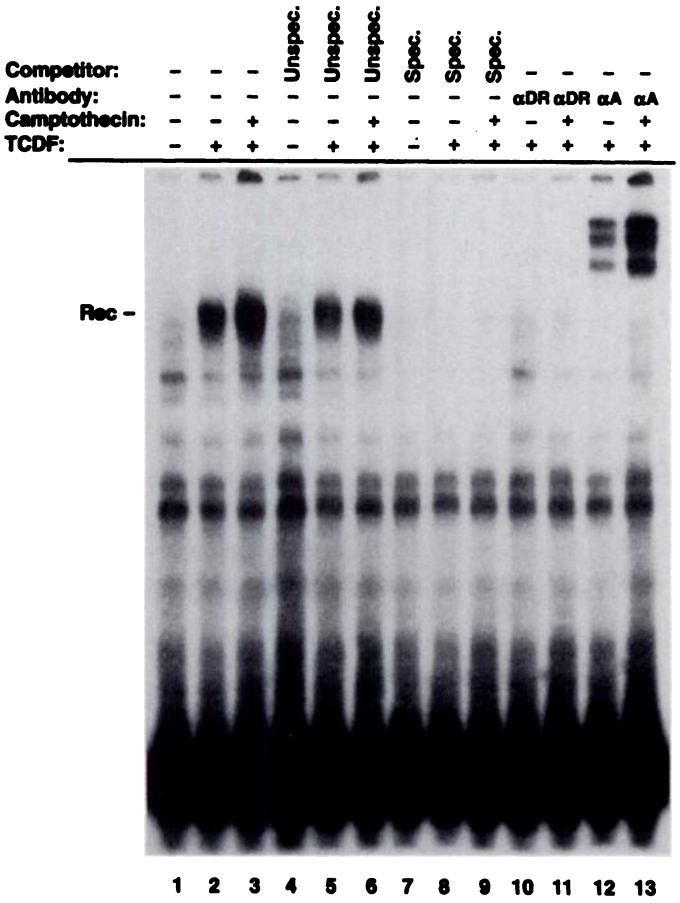

Fig. 3. TCDF-induced accumulation of an XRE-specific DNA binding activity of the dioxin receptor is not affected by camptothecin. *Lanes 1, 4, and 7*, EMSA of nuclear proteins from human keratinocytes treated with DMSO for 1 hr; *lanes 2, 5, 8, 10, and 12*, EMSA of nuclear proteins from human keratinocytes treated with 50 nM TCDF for 1 hr. Cells were treated for 1 hr with 1 μM camptothecin before the addition of 50 nM TCDF for an additional hour (*lanes 3, 6, 9, 11,* and *13*) before harvest. Competition experiments were performed by the addition of a 100-fold molar excess of unlabeled unspecific (*lanes 4–6*) or specific (*lanes 7–9*) oligonucleotide. To identify the XRE-binding proteins, nuclear extracts were preincubated with diluted (1:10) anti-dioxin receptor antiserum (*α-DR, lanes 10* and *11*) or anti-Arnt antiserum (*α-A, lanes 12* and *13*) before the DNA-binding reaction. Note that the anti-dioxin receptor antiserum and the anti-Arnt antiserum recognized a single band (*Rec*).

dioxin-responsive construct containing a dimerized XRE sequence in front of the herpes simplex thymidine kinase promoter and a luciferase reporter gene (30) was also not inhibited by camptothecin treatment (Fig. 4B).

Next, we wanted to determine when during the P450IA1 induction process topoisomerase I activity was required. Keratinocytes were treated with TCDF in the absence or presence of camptothecin or the dioxin receptor ligand ANF for 24 hr (Fig. 5). ANF is a partial agonist for the dioxin receptor and an inhibitor of dioxin action (Ref. 25, including references). When the cells were treated simultaneously with TCDF and either camptothecin or ANF, P450IA1 mRNA levels were not induced (compare *lanes 2–4*). However, if the cells were treated with TCDF for 24 hr before the addition of camptothecin or ANF and incubated for an additional 24 hr, only ANF was capable of suppressing already induced P450IA1 mRNA levels (compare *lanes 6–8*). Thus, once the P450IA1 gene had been induced by dioxin, camptothecin was no longer able to inhibit its transcription.

Discussion

Changes in chromatin structure are known to occur subsequent to binding of the dioxin receptor to target sequences upstream of *CYPIA1*. The importance of these changes are, however, unclear. In the present study, we attempted to modulate this response by inhibiting the major DNA relaxant in the nucleus, topoisomerase I. The topoisomerase I inhibitor camptothecin does not affect activation of the dioxin receptor to its DNA binding form or the transactivating function of the dioxin/receptor complex. Nevertheless, camptothecin treatment can inhibit dioxin induction of two different cytochrome P450 genes, IA1 and IB1.

The mechanism of action of camptothecin leads to the stabilization of a transient intermediate of topoisomerase I covalently bound to DNA via a broken phosphodiester bond in the DNA backbone, thereby inhibiting template relaxation that may be necessary for RNA chain elongation (14). Studies have shown that by inhibiting topoisomerase I, the time course of *fos* induction is delayed (35). However, *fos* mRNA eventually reaches levels similar to those without camptothecin. Because we have examined the effect of camptothecin treatment at a time point (24 hr) that is long after when P450IA1 reaches its maximal level of induction (2–4 hr), this should not interfere with the interpretation of our results.

It is of importance that camptothecin treatment does not affect the expression of *CYPIA1* once gene transcription is induced. It should be noted that prolonged high-level expression is as dependent on new activation of the dioxin receptor as is the initial induction response (Fig. 4) (25). This shows that RNA elongation as such is not severely affected by the treatment. The result can, however, be explained if topoisomerase I activity is needed for chromatin changes associated with dioxin treatment to occur. These changes include appearance of DNase I hypersensitivity over the XRE and the transcriptional start region of *CYPIA1* as well as displacement of a nucleosome in the promoter region (20–22, 37). These effects have not been detected in dioxin receptor-deficient mutant hepatoma cells (24). In this context, it is interesting to note that in another system, a lag period is required for the DNase I hypersensitivity pattern indicative of the repressed state of the gene to re-establish (38). This suggests that rearrangement of chromatin structure is a relatively slow process.

However, dioxin induction of a transiently transfected XRE-containing promoter-reporter gene construct does not show sensitivity to topoisomerase I inhibition. This indicates resemblance to the induction mechanism of the glucocorticoid receptor belonging to the superfamily of nuclear zinc finger-containing receptors. Camptothecin can inhibit expression of glucocorticoid-induced genes such as tyrosine aminotransferase and phosphoenolpyruvate carboxykinase (34). Moreover, it has been shown that glucocorticoid receptor binding to the mouse mammary tumor virus promoter in chromatin configuration leads to structural remodeling allowing for DNA binding of constitutively present factors, including NF-1. In contrast, NF-1 is not excluded from binding to a transiently transfected mouse mammary virus promoter construct (39). In a similar manner, the *CYPIA1* basal promoter may become occupied on exposure to dioxin by the binding of ubiquitous factors belonging to the *Sp1* class (24). It is thus possible that at the point of analysis of transient

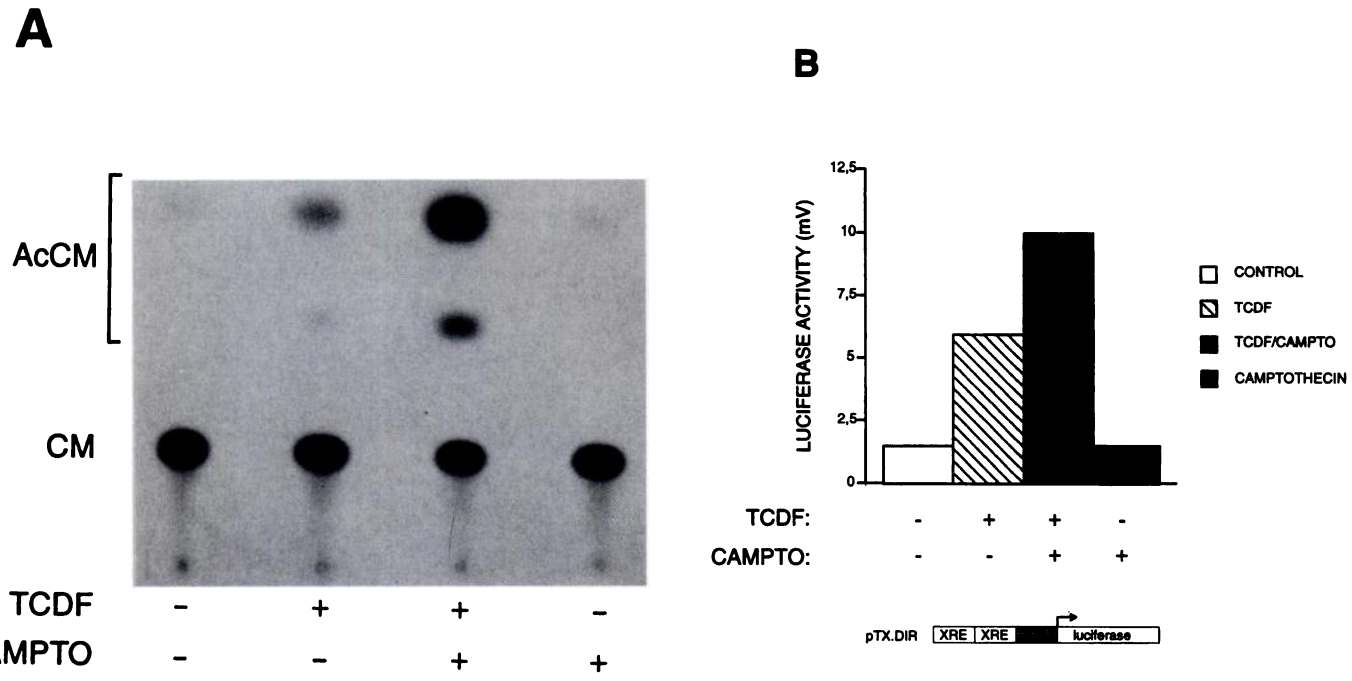

Fig. 4. A transiently transfected XRE containing reporter construct is not affected by camptothecin. A, Human keratinocytes (10 cm^2) were transfected with 1 μg of the phMC7.6 construct that contains a portion of the human *CYPIA1* gene. CAT activity was analyzed by thin layer chromatography after treatment with 50 nM TCDF and/or 1 μM camptothecin (*CAMPTO*). The values have been normalized to the protein content of the cellular extracts. The mobilities of [^{14}C]chloramphenicol (*CM*) and acetylated products (*AcCM*) are indicated. B, Human keratinocytes (10 cm^2) were transfected with 1 μg of the luciferase reporter plasmid driven by an XRE containing minimal thymidine kinase promoter (*pTX.DIR.*). The luciferase activity as analyzed after treatment with solvent alone or 50 nM TCDF in the absence or presence of 1 μM camptothecin. The values have been normalized to the protein content of the cellular extracts and to the activity of the parental construct lacking the XRE sequence (*pT81*). The protein content of extracts from cells treated with camptothecin was not significantly different from that of control cells. *Bars*, average values obtained from three independent experiments.

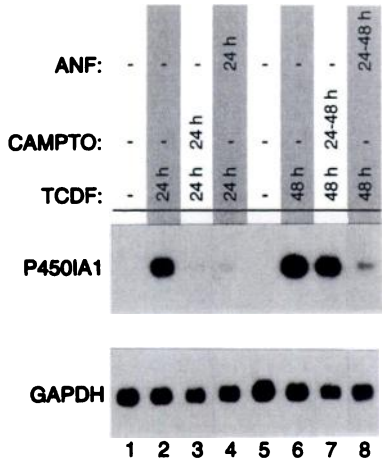

Fig. 5. A topoisomerase I inhibitor does not inhibit maximally induced P450IA1 levels. RNA blot analysis of total RNA prepared from human keratinocytes treated either with solvent (DMSO) alone or with 50 nM TCDF in the absence or presence of simultaneously added 1 μM camptothecin (*CAMPTO*) or 10 μM ANF for 24 hr (*lanes 1–4*). *Lanes 5–8*, cells were incubated with 50 nM TCDF for 24 hr before the addition of 1 μM camptothecin or 10 μM ANF for an additional 24 hr. Note that treatment with the competitive dioxin receptor antagonist ANF supressed already induced P450IA1 levels, whereas camptothecin did not. Cytochrome P450IA1 and control GAPDH mRNA levels were analyzed.

transfections, the promoter is already occupied by the appropriate factors, thus eliminating the need for chromatin changes elicited by dioxin treatment.

Rather than inhibiting the dioxin induction response, camptothecin can increase expression from transiently transfected XRE-containing reporter constructs. This may be due to enhanced expression of extrachromosomal plasmid in the absence of topoisomerase I, probably due to accumulation of negative supercoils that promote RNA polymerase II binding to promoter sequences, as has been described for mutant yeast strains (40). In addition, this result suggests that the role of the dioxin receptor is not solely to bring about the observed changes in *CYPIA1* chromatin structure but rather that it fulfills an important transactivating function. However, it is an intriguing possibility that the dioxin receptor may predominantly fulfill a "chromatin opener" role for other response genes.

In summary, we have shown that topoisomerase I activity is needed for dioxin-mediated activation of the cytochrome P450IA1 gene but not for dioxin-dependent continued high-level expression. One possibility is that the noninduced *CYPIA1* gene is present in a specific structural state that requires topoisomerase activity for initiation of RNA polymerase II activity. Although topoisomerase I has been implicated to be a component of the basal transcription machinery, possibly acting as a cofactor for activated transcription levels, its precise role in transcription remains unclear (41, 42). We favor the model that topoisomerase I activity is needed for structural changes to occur in the target P450IA1 promoter region.

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